

Isolation and Characterization of Inner and Outer Membranes of *Xanthomonas campestris* pv. *campestris*

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This investigation was a project under NC-135 and NCR-100 and was supported in part by grants to N. W. Schaad from the Garden Seed Research Committee of the American Seed Research Foundation, Georgia Transplant Growers Association, and the U.S. Department of Agriculture, and by Hatch Project 1228. J. C. Dianese was supported in part by a sabbatical fellowship from the National Research Council of Brazil (CNPq).

We thank R. E. Wilkinson for helping in the identification of phospholipids, and F. C. Boswell for the phosphate analysis.

Accepted for publication 10 March 1982.

ABSTRACT

Dianese, J. C., and Schaad, N. W. 1982. Isolation and characterization of inner and outer membranes of *Xanthomonas campestris* pv. *campestris*. *Phytopathology* 72:1284-1289.

Cell envelopes of *Xanthomonas campestris* pv. *campestris* were extracted by conventional methods and characterized. The total membrane fraction was resolved into a light (L), intermediate (M), and two heavy (H₁ and H₂) fractions by 45–70% sucrose step density gradient centrifugation. The L fraction contained 67% of the succinate dehydrogenase (SDH) activity, whereas the H₁ and H₂ fractions contained 69% of the 2-keto-deoxyoctonate (KDO). Most remaining SDH (18%) and KDO (25%) was in the M fraction. Xanthomonadin, a brominated arylopolypene pigment, was located exclusively in fractions H₁ and H₂. Based on these data, fractions L and H₁ plus H₂ were considered to be primarily compounds from the inner and outer membranes, respectively. The relative phospholipid content of

the inner membrane was considerably higher than that of the outer membrane. Six phospholipids were identified; the bulk (about 20% each) of these were lysophosphatidylethanolamine, phosphatidylethanolamine, and phosphatidylserine. Electron micrographs showed the inner membrane to consist of circular unit membranes much smaller than the larger elongated structures of the outer membrane. Approximately 30 polypeptides in the total membrane fraction were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Three major polypeptides (44, 26, and 23 kilodaltons) were resolved in the outer membrane fraction. The possible importance of membrane proteins in the pathogenicity of *X. campestris* is discussed.

The cell envelope of Gram-negative bacteria consists of an inner cytoplasmic membrane and an outer membrane composed of a layer of peptidoglycan between the two distinct membranes (4). Both membranes contain phospholipids and proteins. The inner membrane contains several specific enzymes, whereas the outer membrane contains lipopolysaccharide (LPS) and several major proteins such as porins and lipoproteins (13,36). Much information is available on membranes of members of the Enterobacteraceae and organisms of medical importance (3,12,14,27–29,35,39), but little is known about the membranes of plant pathogenic bacteria.

Shukla and Turner (44) isolated and characterized inner and outer membranes of *Erwinia carotovora*, a soft rotting member of the Enterobacteraceae. The membranes obtained by sonic disruption of spheroplasts produced by a lysozyme/EDTA treatment were characterized by using 2-keto-deoxyoctonate (KDO) content and succinate dehydrogenase (SDH) activity of the two resulting density gradient fractions. Total membrane proteins of *Xanthomonas sinensis* have been analyzed by SDS-PAGE (40), but no attempt was made to separate inner and outer membranes.

Lipopolysaccharides of Gram-negative plant pathogenic bacteria have been implicated as the site of molecular interaction with cell walls or membranes of host plants (9,22–24,30,43,50). A better knowledge of the components of the bacterial cell envelope might contribute to a better understanding of such molecular interactions.

Xanthomonas campestris pv. *campestris* (called *X. campestris* in this article), a member of the Pseudomonadaceae, is the causal agent of black rot of crucifers and is the most destructive pathogen of crucifers worldwide (53). Furthermore, this organism is of major

importance to food processing, textile, and oil well drilling industries because it produces xanthan gum, an extracellular polysaccharide (51). We are interested in the possibility that cell envelope components might be involved in limiting the pathogenicity of *X. campestris* to plants in the family Cruciferae. To pursue this, it was necessary to isolate and separate the component membrane fractions.

In this paper, we describe a procedure for separating the cytoplasmic and outer membrane of *X. campestris*. Data are presented on some of the chemical components, selected enzyme activities, and polypeptide composition.

MATERIALS AND METHODS

Bacterial growth and envelope isolation. *X. campestris* strain B-24 (obtained originally from L. M. Moore in Oregon in 1971) was maintained by weekly transfers on slants of YDC medium (54). Cultures were stored on YDC slants at 2 C with transfers every 2–3 mo. A seed culture of the bacterium was grown in liquid medium 523 (16) on a rotary platform shaker at 30 C. After 15 hr, 15 ml of seed culture were added to 1.5 L of 523 medium in 2.8-L Fernbach flasks. The flasks were shaken at 50–60 rpm on a New Brunswick (model G-25) rotary shaker at 30 C for 20–24 hr.

Bacterial cells were harvested during mid-logarithmic growth (100–150 Klett units) by centrifugation at 16,300 g for 10 min at 4 C, washed once with 10 mM tris (tris[hydroxymethylamino-methane]-HCl buffer pH 7.4 (tris buffer), and resuspended in the same buffer (100 ml per 10–15 g of cells). DNase (Sigma Chemical Co., St. Louis, MO 63178) and RNase (Worthington Biochemical Corp., Worthington, OH 43085) at 14 and 15 units per milliliter, respectively, were added and the cell suspension was stirred for 30 min at 4 C. The cells were broken by a single passage through a chilled French pressure cell (American Instrument Co., Silver Spring, MD 20910) at 124 to 138 MPa (18,000 to 20,000 psi). The lysate was centrifuged at 3,000 g for 10 min, and the pellet

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containing unbroken cells was discarded. Total cell envelopes were obtained from the supernatant fluid by centrifugation at 190,000 g for 90 min in a Type 42.1 Beckman rotor. The resulting cell envelopes were washed once, suspended in 2–4 ml of tris buffer, and stored in 15–20 ml vials at –20 C or used fresh.

Sucrose density gradient centrifugation. Total cell envelopes were layered on a step density gradient similar to that of Hancock and Nikaido (11), but with the following sucrose composition in tris buffer (w/w): 5 ml of 45%, 8 ml of 52%, 9 ml of 58%, 10 ml of 64%, and 3 ml of 70%. Total envelopes (0.5–1.0 ml) containing 12–15 mg of protein per milliliter were layered onto each of six gradients and centrifuged at 120,000 g in a Beckman SW 27 rotor for 18 hr at 4 C. The gradients were fractionated by using an ISCO (Instrumentation Specialties Co., Lincoln, NE 68504) gradient fractionator and UA-5 automatic peak separator and an ISCO model 568 fraction collector. Each membrane fraction was isolated by pooling fractions under each peak, dialyzing at 4 C for 15–18 hr against 3 L of tris buffer (with 3 changes), and centrifugation at 190,000 g for 90 min. The membrane pellets were washed once and suspended in 1–2 ml tris buffer overnight on a platform shaker at 4 C. Preparations were stored at –20 C or used fresh. Only unfrozen material was used for enzyme assays.

Densities of fractions were estimated by measuring the sucrose concentration with a Bausch & Lomb refractometer at 20 C from 1 ml gradient fractions.

Chemical and enzyme assays. Protein was analyzed by following the procedures of Lowry et al (25). The KDO content was estimated by the thiobarbituric acid method (49). The final chromogen was extracted with an equal volume of butanol containing 5% HCl, and differences in absorbance at 552 and 508 nm were recorded; the amount of KDO was determined by using a micromolar extinction coefficient of 19 (19).

Phospholipids were extracted by following the procedures of Folch et al (7). The chloroform-methanol (2:1, v/v) extract was evaporated under minimum light, and the lipid fraction was dissolved in chloroform. These samples were stored at –20 C under nitrogen. Xanthomonadins (47) were estimated by the absorbance at 453 nm of the lipid extract in chloroform (1) using a molar extinction coefficient of 131,500 (1). Phospholipid species were identified by thin-layer chromatography (20) with 0.5 mm silica gel G plates treated with 0.15 M (NH₄)₂SO₄ and activated for 1 hr at 110 C. Plates were cooled to room temperature in a desiccator. Chromatography was performed in paper-lined saturated chambers using acetone:benzene:H₂O (91:30:8, v/v) solvent. The phospholipid species were located by ultraviolet light after the plates were sprayed with a fluorescent mixture (15). Spots were eluted with chloroform-methanol and phosphate was determined (31).

Nicotinamide adenine dinucleotide (NADH) oxidase was measured as described (34) in reaction mixtures containing 50 mM tris-HCl, pH 7.5, 0.12 mM NADH (Sigma), 0.2 mM dithiothreitol (Sigma), and 150 µg of freshly prepared envelope protein. Decrease in absorbance at 340 nm was measured for 20 min at 30 C. Succinate dehydrogenase (SDH) was assayed according to Kasahara and Anraku (18) in reaction mixtures (3 ml) containing 50 mM tris-HCl, pH 8.0, 4 mM KCN, 0.04 mM 2,6-dichlorophenol-indophenol (DCPIP) (Sigma), 0.2 mM phenazine methosulfate (Sigma), 30 mM potassium succinate (United States Biochemical Corporation, Cleveland, OH 44128), and 100–200 µg of cell envelope protein. Decrease in absorbance at 600 nm was measured for 10 min at 30 C. Mannanase activity was measured as specified by Dekker and Candy (4) on 1 ml of reaction mixture containing 0.5% galactomannan polymer (Sigma) and 1 mg membrane protein in tris buffer. The mixture was incubated for 30 min at 30 C before reducing sugars were measured by the copper/arsenomolybdate method (33). All absorbance readings were determined with a Gilford model 220 photometer and Beckman DU monochromator attached to a Heathkit EU-205-11 recorder.

Electron microscopy. Samples for negative staining were deposited on Parlodion (Parlodion, St. Louis, MO 63147)-coated grids and stained with 2% aqueous uranyl acetate for 2 min. Samples for sectioning were centrifuged at 195,000 g for 1 hr in

Spinco type 40 polycarbonate centrifuge tubes. The pelleted membranes were fixed in 4% glutaraldehyde for 15 hr, washed, postfixed in 2% osmium tetroxide, and embedded in Spurr's medium (46). Thin sections (silver to gold) were stained in 2% aqueous uranyl acetate and 1% lead citrate, and viewed under a Phillips model 200 electron microscope operated at 80 kV.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in vertical slab gels of 10% acrylamide on a Bio-Rad model 221 electrophoresis apparatus with the discontinuous system of Laemmli (21). Samples were solubilized in Laemmli sample buffer (21) at 100 C for 3 min. Ten to 20 µl of sample containing 400 µg of protein per milliliter was applied to each sample well and electrophoresis was performed at 15 C with a constant current of 12.5 ma regulated by an ISCO model 1420 power supply. Molecular weights were determined by using a Bio-Rad low-molecular-weight standard protein solution (48).

RESULTS

Isolation of membrane fractions. Sucrose density gradient centrifugation of cell envelopes resulted in four distinct peaks (Fig. 1). Buoyant densities of the light (L), intermediate (M), heavy 1 (H₁), and heavy 2 (H₂) fractions were 1.143, 1.163, 1.186, and 1.193 g/cm³, respectively.

Chemical composition of the isolated membrane fractions. The chemical composition of membrane fractions is shown in Table 1. Nearly half of the protein recovered from the gradient fractions was present in fraction H₁; the remaining protein was evenly distributed between the other fractions. However, the highest concentration of protein per milligram (dry weight) of membrane was in fraction L, which proved to be the cytoplasmic membrane (see below). Xanthomonadin was present only in fractions H₁ and H₂. Fractions M, H₁, and H₂ were enriched in KDO, whereas fractions L and M were greatly enriched in phospholipids. Six phospholipids, including cardiolipin (CL), lysophosphatidylethanolamine (LPE), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS) were tentatively identified in each of the fractions with LPE, PI, and PS predominating (Table 2).

Enzyme activities associated with the isolated membrane fractions. The distribution of enzyme activities in the isolated

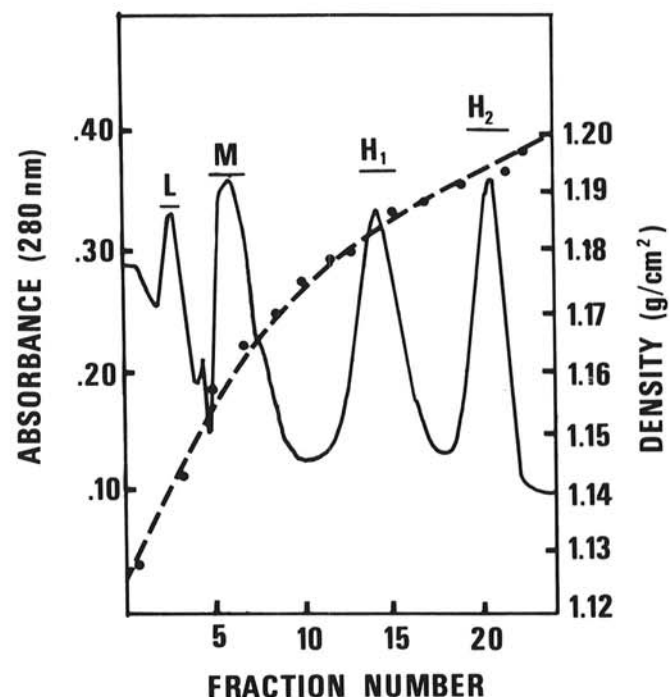


Fig. 1. Separation of membranes of *Xanthomonas campestris* B-24 on 52–70% step sucrose density gradients. Gradients were centrifuged at 120,000 g for 18 hr at 4 C.

membrane fractions is shown in Table 3. Fractions L and M together contained 85, 72, and 65% of SDH, NADH oxidase, and β -mannanase activity (per milligram dry weight membrane), respectively. Fraction L contained 67% of the SDH activity (per milligram [dry weight] of membrane), whereas fraction H₁ contained only 3%.

Electron microscopy. Negative staining revealed membrane structures in each of the four gradient band samples. Those in band L and M were circular, approximately 30–50 nm in diameter and of uniform size, whereas those in band H₂ were mostly elongate, approximately 150 nm wide and 220–500 nm long (Fig. 2A–C). There was little difference between structures in H₁ and H₂ except that those in H₂ appeared slightly larger. Sectioned material from band H₂ showed uniform-shaped bilaminar unit membrane structures (Fig. 2D).

SDS-polyacrylamide gel electrophoresis. Polypeptide profiles of membrane fraction L were significantly different from fractions H₁ and H₂ (Figs. 3 and 4). Fraction L contained several minor polypeptides, but no major ones (Fig. 3, lane 1). On the other hand, fractions H₁ and H₂, which were undistinguishable, contained major polypeptides of 44, 26, and 23 kilodalton (kda) with the 44 kdalton polypeptide predominating (Fig. 3, lanes 3 and 4, and Fig. 4). Profiles of H₁ and H₂ fractions contained more minor polypeptides than did the L fraction. Fraction M contained the three major polypeptides observed in fraction H₁ and H₂ but in smaller amounts (Fig. 3, lane 2).

DISCUSSION

We have described a procedure useful for characterizing the membranes of *X. campestris*, a Gram-negative plant pathogenic

bacterium. At first we employed the EDTA-lysozyme methods used to prepare membranes of *E. coli* (29) and *S. typhimurium* (35). However, inner and outer membranes of *X. campestris* failed to separate adequately by either method. Our results are in agreement with the failure of the EDTA-lysozyme method to separate membranes of *Pseudomonas aeruginosa* (11) and *Selenomonas ruminatum* (17). Sucrose step density centrifugation of envelopes of *X. campestris* from French pressure cell extracts results in four fractions. From determinations of chemical composition and enzyme activities, we found that the L fraction and the heaviest fraction (H₂) were enriched in cytoplasmic and outer membranes, respectively. The H₂ band contains a relatively small amount of enzyme activity (9%) and a relatively large amount of KDO (44%), whereas the L band contains a relatively small amount of KDO (8%) and a relatively large amount of enzyme activity (54%). The M and H₁ bands contain a moderate amount of enzyme activity (21 and 17%, respectively) and outer-membrane-specific KDO (23 and 25%, respectively), suggesting a mixture of cytoplasmic and outer membrane.

The appearance of two sucrose density gradient fractions enriched in outer membrane has been reported for the closely related *P. aeruginosa* (11) when similar extraction procedures were used. However, fraction H₁ of *X. campestris* is less enriched in outer membrane than fraction H₂. Whether this difference is due to the extraction procedure (such as 124–138 MPa [18,000–20,000 psi] versus 103 MPa [15,000 psi] for cell breakage) or to the structure of the outer membrane of *X. campestris* is unknown. The difference between the H₁ and H₂ fractions may be a result of differential amounts of KDO and xanthomonadin, a yellow brominated arylopolypene pigment produced only by xanthomonads (47). The

TABLE 1. Chemical composition of isolated membrane envelope fractions of *Xanthomonas campestris* B-24

Fraction	Buoyant density (g/cm ³)	Recovery of protein (% total) ^a	Composition			
			Protein ^b	Phospholipids ^b	Xanthomonadin ^c	KDO ^c
Total membrane	276	55	0.38	2.20
Light (L)	1.143	13.9	574	145	0.00	0.68
Intermediate (M)	1.163	17.5	314	106	0.00	2.26
Heavy 1 (H ₁)	1.186	49.9	284	38	0.78	2.04
Heavy 2 (H ₂)	1.193	18.7	408	29	0.51	3.98

^aRepresents distribution of protein within the sucrose density gradient fractions.

^bMicrograms per milligram (dry weight) of membrane.

^cNanomoles of xanthomonadin or 2-keto-3-deoxyoctonate (KDO) per milligram of protein.

TABLE 2. Amounts and distribution of phospholipids identified in membrane envelope fractions of *Xanthomonas campestris* B-24

Fraction ^a	Phospholipids (μ g/ml of membrane) ^b						
	CL	LPE	PE	PG	PI	PS	U
TM	3.2 ^c	11.3	3.2	8.1	8.1	12.9	8.1
L	8.1	24.1	16.1	16.1	36.3	28.1	16.2
M	4.8	32.9	12.8	12.9	32.6	31.0	16.2
H ₁	1.6	11.0	3.2	2.3	10.0	7.3	2.7
H ₂	0.8	6.7	2.9	3.4	9.4	5.0	3.4

^aAbbreviations (fractions): TM, total membrane; L, light; M, intermediate, and H, heavy.

^bAbbreviations (phospholipids): CL, cardiolipin; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; and U, unknown.

^cThe distribution of total phospholipid is shown in Table 1.

TABLE 3. Enzyme activities of isolated membrane fractions of *Xanthomonas campestris* B-24

Enzyme	Specific activity ^a				
	Total membrane	Light (L)	Intermediate (M)	Heavy 1 (H ₁)	Heavy 2 (H ₂)
Succinate dehydrogenase	5.0	34.1 (59.4)	5.0 (15.9)	3.0 (10.6)	1.0 (2.5)
NADH oxidase	7.4	15.0 (26.1)	7.4 (23.6)	4.1 (14.4)	2.1 (5.1)
β -mannanase	3.5	25.7 (44.8)	3.7 (11.8)	4.6 (16.2)	5.8 (14.2)

^aSpecific activities are expressed in micromoles substrate oxidized per minute per milligram of protein. Numbers in parenthesis are specific activities per milligram (dry weight) of membrane (based on 574, 314, 284, and 408 μ g of protein per milligram [dry weight] of membrane of fractions L, M, H₁ and H₂, respectively).

absence of xanthomonadin in the KDO containing M fraction suggests that the pigment is located in a layer of the outer membrane that is different from that where LPS is located. This suggests that the inner and outer membranes of *X. campestris* separate by layers. Our results establish that xanthomonadin is limited and exclusive to the outer membrane. Because the pigment is easy to measure, it may be used as an excellent outer membrane marker for yellow-pigmented xanthomonads.

Because only one solvent system was employed, our identification of six classes of phospholipids is not definitive. In fact, the presence of an unidentified phospholipid that is 11% of the total suggests incomplete identification. Furthermore, the relatively high level of LPE in membranes of *X. campestris* suggests that PE might have been hydrolyzed by degradative enzymes during the assay procedure (2). PE has been identified as a major phospholipid in inner and outer membranes of *Acinetobacter* (42), *E. coli* (52), *Proteus mirabilis* (8,38), *S. typhimurium* (35), and in whole cell extracts of *Neisseria gonorrhoeae* (10). A larger amount of each phospholipid in the inner membrane of *X. campestris* agrees with data which indicate that inner-membrane-bound enzymes are responsible for the synthesis of bacterial phospholipids (2).

SDS-PAGE profiles of SDS- and heat-solubilized membrane proteins of *X. campestris* differ from profiles of most other bacteria that have been studied. Most of those studied, including *S. typhimurium* (35), *E. coli* (39,41), *S. ruminantium* (17), *Pseudomonas aeruginosa* (11,28), and *Rhodospirillum rubrum* (3) contain considerably more polypeptides in the inner membrane than in the outer membrane. However, such is not true for *X. campestris*. The reason for this could be due to the relatively slow growth of the plant pathogen in liquid medium, solubility of the polypeptides of the inner membrane, or loss of inner membrane polypeptides. Perhaps, since the organism is a pathogen specific to plants of a single family (Cruciferae), fewer enzymes are needed. In addition, the nutritional range of *X. campestris* is quite limited (6)

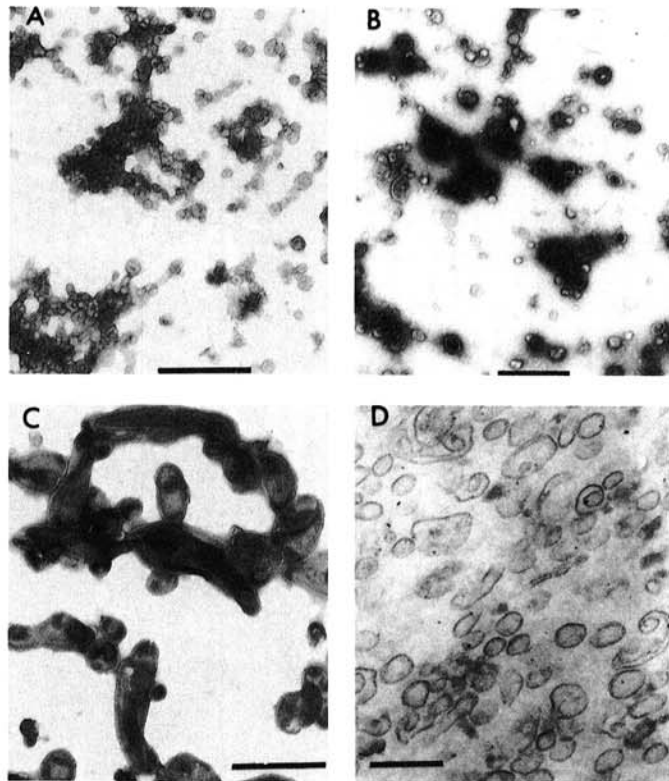


Fig. 2. Electron micrographs of fragments of membranes of *Xanthomonas campestris* B-24 from sucrose density gradient centrifugation bands. Negatively stained samples of A, L; B, M; and C, H₂; and D, sectioned material from H₂. Scale bars for A, B, and C = 0.43 μ m. Scale bar for D = 0.25 μ m.

whereas that of *P. aeruginosa*, a closely related organism, is very diverse (37).

The profile of the major outer membrane polypeptides of *X. campestris* is very similar to that of *P. aeruginosa*. Both organisms contain three major polypeptides when compared under similar conditions (11). The 44, 26, and 23 kdaltons polypeptides of *X. campestris* are each approximately 5 kdaltons higher than the three major polypeptides of *P. aeruginosa* (11). Perhaps such a pattern of major outer membrane polypeptides is unique to the Pseudomonadaceae. A major similarity between the profiles of *X. campestris* and many other Gram-negative bacteria is the presence of a 44 kdaltons heat-modifiable (26) major polypeptide similar to the OmpA polypeptide (5,13,32) of other Gram-negative bacteria.

Just as cell envelope components of animal pathogens have a major role in determining pathogenicity (45), cell envelope components of plant pathogenic bacteria may also play a role. A

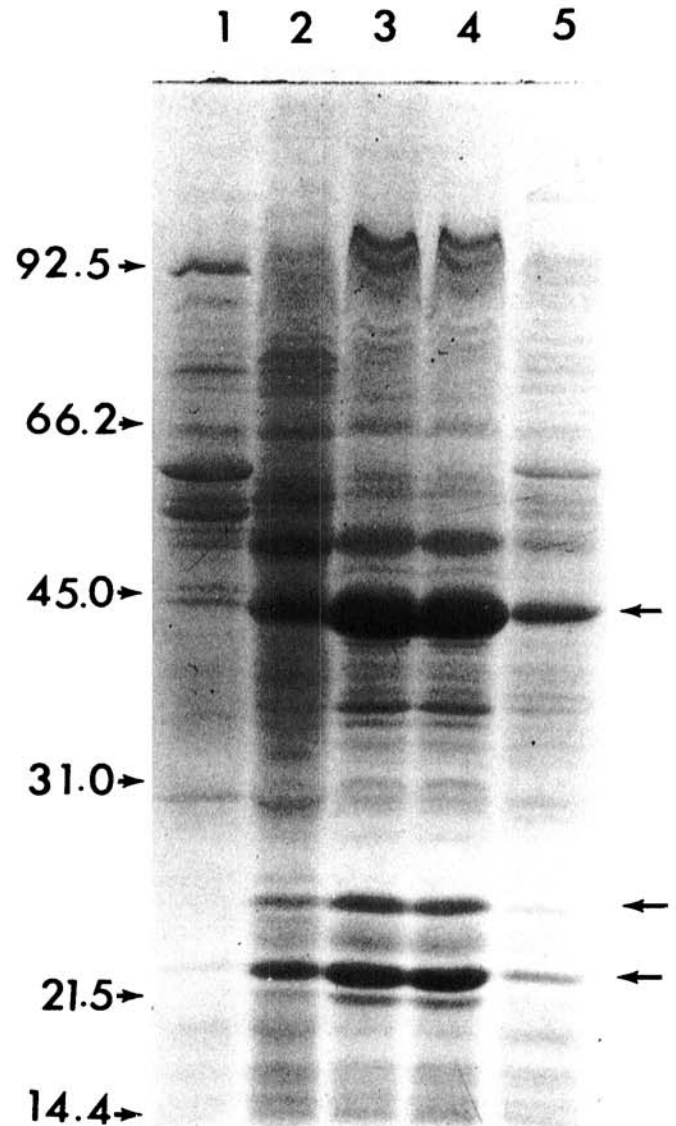


Fig. 3. Coomassie brilliant blue-stained SDS-polyacrylamide gel electrophoresis profile of membrane fractions of *Xanthomonas campestris* B-24. The fractions were prepared as described in Materials and Methods. Electrophoresis was performed in vertical slab gels of 10% polyacrylamide with the discontinuous system of Laemmli (27). Each fraction preparation was solubilized in Laemmli sample buffer at 100 C for 3 min. Samples were sucrose density gradient centrifugation fractions: 1, light; 2, intermediate; 3, heavy (H₁); and 4, heavy (H₂); and 5, total membrane. Numbers on the left (marked by arrows) are molecular weights ($\times 10^3$). Eight micrograms of protein were applied to each lane. Major polypeptides are noted by arrows on the right.

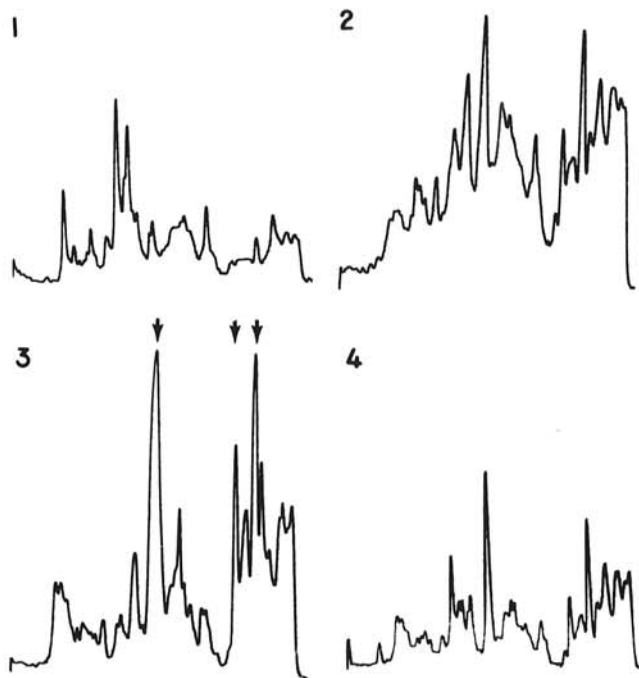


Fig. 4. Scans of SDS-polyacrylamide gels shown in Fig. 3. Scans are of sucrose density gradient fractions: 1, light; 2, intermediate; 3, heavy (H_2); and 4, total membrane. Major polypeptides of outer membrane are noted by arrows. Gels were scanned at 520 nm by using a photovolt densitometer.

better understanding of the biochemistry and structure of bacterial membranes is certainly needed before fruitful advances can be made toward understanding the interactions between plant pathogenic bacteria and plant hosts at the molecular level. The methods we have described can be successfully used to fractionate the cell envelope of *X. campestris* B-24 into four fractions. Fraction H_2 , representing almost-pure outer membrane, will be useful in our planned studies on the role of outer membrane proteins in pathogenesis.

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